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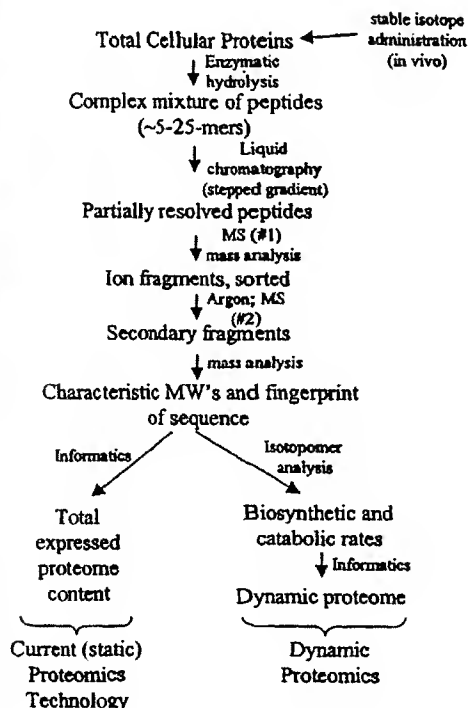
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(54) Title: METHOD FOR AUTOMATED, LARGE-SCALE MEASUREMENT OF THE MOLECULAR FLUX RATES OF THE PROTEOME OR THE ORGANEOME USING MASS SPECTROMETRY



(57) Abstract: Disclosed here is a method for measuring the kinetics (i.e., the molecular flux rates - synthesis and breakdown or removal rates) of a plurality of proteins or organic metabolites in living systems. The methods may be accomplished in a high-throughput, large-scale automated manner, by using existing mass spectrometric profiling techniques and art well known in the fields of static proteomics and static organomics, without the need for additional biochemical preparative steps or analytic/instrumental devices.

Technique for Measuring Dynamic Proteomics



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**METHOD FOR AUTOMATED, LARGE-SCALE MEASUREMENT
OF THE MOLECULAR FLUX RATES
OF THE PROTEOME OR THE ORGANEOME USING MASS SPECTROMETRY**

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. patent application 60/399,950 filed on July 30, 2002 which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to methods for measuring molecular flux rates (synthesis and breakdown or input and removal rates from pools of molecules) in the proteome and the organeome (dynamic proteomics and dynamic organeomics, respectively) using mass spectrometry. The methods disclosed are capable of high-throughput, large-scale, automated applications. The methods are applicable to studies in genetics, functional genomics, drug discovery and development, drug toxicity, clinical diagnostics and patient management.

BACKGROUND OF THE INVENTION

Recent advances in the Human Genome project have, paradoxically, led to the wide-spread recognition of the inadequacy of gene sequence information by itself. Sequence information (i.e. structural genomics) is unlikely to generate insight into disease or normal physiology without better information concerning the functional consequence of genes. Higher levels of biological organization relative to gene sequences include expressed mRNA levels, the expressed protein complement and concentrations of organic molecules in metabolic pathways. These levels of cellular organization have been called gene expression profiling (transcriptomics), proteomics and organeomics, respectively. In aggregate, these can be seen as including the structural biochemical phenotype (i.e. the complete complement of molecules present) in a cell or organism.

Gene expression profiling of mRNA has been achieved through the development of gene expression chips. Such chips are available from companies such as Affymetrix. Enumerating the expressed genome (i.e. the complement of mRNA species), even in its entirety, however, does not ultimately provide information about biochemical function (phenotype) in a living system. Although impressive as a technology, gene expression chips do not solve the central problems of phenotype and function in biochemistry, which relate to the flow of molecules through the complex interactive network of proteins that comprise fully assembled living systems.

Other methods have focused on characterizing the complement of proteins in a living system, i.e. the "proteome." The most powerful technology for automated, large-scale characterization of expressed proteins (proteomics) to date has proven to be mass spectrometry. Mass spectrometers have greatly simplified large-scale automated proteome analysis. Analogous mass spectrometric methods have been advanced for the automated, large-scale characterization of organic metabolites (organeomics).

Many scientists in the pharmaceutical industry, including those in genomics companies, are predicting a log-jam of potential drug leads and targets that are under development. This log-jam arises from bottle-necks in the testing of phenotypic consequences of inhibiting particular targets – i.e. the "tail-end" of drug development. The tail-end of drug development (target validation) has not received a similar push from breakthrough technologies as the "front-end" (target identification and identification of chemical modulators of targets) of drug discovery.

One of the central problems in this area relates to the absence of routine, high-throughput dynamic measurements in biology and medicine. Just as biochemical phenotype is recognized to be reducible to the flow of molecules through metabolic pathways in complex catalytic networks, it is also widely recognized that most diseases reduce to an altered rate of a normal process. For example, atherogenesis reflects vascular wall proliferation and uptake of lipids; carcinogenesis reflects cell proliferation; infection can be characterized as microbial division, growth and death – this formulation is more informative than describing these disorders as alterations of static measures (e.g. concentrations of cholesterol, carcinogens, or bacteria). Yet rarely, if ever, are

rates of biochemical processes measured in medical diagnostics. Static markers of dynamic processes are often helpful and may be better than nothing, but they are not the true measure of disease activity or disease risk. Nor do static measures allow for personalized biochemical monitoring. For example, each individual may have a different relationship between CD4 count and true turnover of T lymphocytes in HIV infection, or between DNA-adducts and the true risk of cancer, or between LDL cholesterol and the true rate of atherogenesis. In the final analysis, kinetic questions must be addressed by direct kinetic measurements.

Thus, the current art in mass spectrometric proteomics and organeomics is characterized by a shared and fundamental limitation: the information is static, not dynamic. Missing from both static proteomics and static organeomics is kinetics: fluxes into and out of the pools of molecules that are present in the system. Kinetics or dynamics differ from statics in the fundamental respect that the dimension of time is included. Kinetics refers to the study of time-related changes in molecules whereas the concentrations of proteins or organic molecules determined in static measurements do not provide any information about their rates of change over time. Although the current techniques of static proteome and organeome characterization can provide a snapshot of what is present, these techniques cannot provide information concerning flows of molecules through the system (kinetics).

Thus, there is a tremendous need for the large scale determination of molecular flux rates of a plurality of proteins or organic metabolites – i.e. "dynamic proteomics" and "dynamic organeomics".

SUMMARY OF THE INVENTION

In order to meet these needs, the present invention is directed to a method of determining the molecular flux rates (i.e., the rates of synthesis or breakdown of a plurality of proteins in all or a portion of the proteome of a cell, tissue or organism). One or more isotope-labeled protein precursors are administered to a cell, tissue or organism for a period of time sufficient for one or more isotope labels to be incorporated into a plurality of proteins in the proteome or portion thereof of the cell, tissue or organism. The proteome or portion thereof are then obtained from the cell,

tissue, or organism. A plurality of mass isotopomeric envelopes representing individual proteins in the proteome or portion of the proteome are then identified by mass spectrometry. In addition, the relative and absolute mass isotopomer abundances of the ions within the isotopomeric envelope corresponding to each identified protein are quantified by mass spectrometry. These relative and absolute mass isotopomer abundances allow the molecular flux rates of each identified protein to be calculated and the molecular flux rates of the plurality of proteins thereby to be determined.

In one aspect, the administering step may be continuous. The protein precursors may also be administered at regular measured intervals. The protein precursors may also be administered orally. The method may include the additional step of discontinuing the administering step.

The one or more protein precursors may be an amino acid, or may include one or more precursors such as H_2O , CO_2 , NH_3 , and HCO_3 . Isotope label may include one or more isotopes such as ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S and ^{34}S . In a particular embodiment, the isotope label may be ^2H .

In another aspect, the proteins may be modified prior to the measuring step. The modification may be by any method known in the art, such as biochemically degrading the proteins, or by chemically altering the proteins.

In a further aspect, the individual proteins may also be identified by both chromatography and mass spectrometry. In an additional embodiment, the plurality of proteins may also include the entire proteome of the cell, tissue, or organism. The calculated molecular flux rates of the proteins may be displayed after calculation.

The organism may be any organism in the art from cells in culture to living animals. In one aspect, the organism is a human.

In another aspect, the methods may further include administering a diagnostic or therapeutic agent to the cell, tissue, or organism prior to administering the isotope labeled precursor. The invention is also directed to a method of determining the effect of a diagnostic or therapeutic agent on a cell, tissue, or organism, by determining the molecular flux rates of a plurality of proteins in the cell, tissue, or organisms, administering the agent and again determining the molecular flux rates of the plurality

of proteins in the cell, tissue or organism. The discovery and development of drugs can be achieved or facilitated by this means.

The methods may also include determining the effects of one or more genes on the molecular flux rates of synthesis of a plurality of proteins in a cell, tissue, or organism by determining the molecular flux rates of a plurality of proteins in a first population of one or more cells, tissues, or organisms that has one or more genes, determining the molecular flux rates on the plurality of proteins in a second population of one or more cells, tissues, or organisms that does not have the one or more genes, and comparing the molecular flux rates in the first and second populations to determine the effect of one or more genes on the molecular flux rates of a plurality of proteins.

In another aspect, the invention is drawn to determining the molecular flux rates of a plurality of organic metabolites in all or a portion of the organeome of a cell, tissue or organism. One or more isotope-labeled organic metabolites or organic metabolite precursors are administered to the cell, tissue or organism for a period of time sufficient for one or more isotope labels to be incorporated into a plurality of organic metabolites in the organeome or portion thereof of the cell, tissue or organism. The organeome or portion thereof is obtained from the cell, tissue, or organism. A plurality of mass isotopomeric envelopes of ions representing individual organic metabolites in the organeome or portion thereof are identified by mass spectrometry. In addition, the relative and absolute mass isotopomer abundances of the ions within the isotopic envelopes corresponding to each identified organic metabolite are quantified by mass spectrometry. These relative and absolute mass isotopomer abundances allow the rates of synthesis or removal of each identified organic metabolite to be calculated, and the molecular flux rates of the plurality of organic metabolites thereby to be determined.

In one aspect, one or more organic metabolites or organic metabolite precursors include one or more of H_2O , CO_2 , NH_3 , HCO_3 , amino acids, monosaccharides, carbohydrates, lipids, fatty acids, nucleic acids, glycolytic intermediates, acetic acid, and tricarboxylic acid cycle intermediates. In another aspect, the isotope label includes ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S or ^{34}S . The plurality of organic metabolite precursors may include the

entire organeome. The organism may be any known organism, including a human. In a particular embodiment, the isotope label may be ^2H .

In another aspect, the administration of precursors may be continuous. Alternatively, the precursor may be administered at regular measured intervals. The one or more organic metabolites or organic metabolite precursors may be administered orally. Further, the method may include the additional step of discontinuing administration of the labeled precursor.

In an additional aspect, the method may include modifying organic metabolites prior to introduction into the mass spectrometer. The modification may be any method known in the art, such as biochemically degrading the organic metabolites or chemically altering the organic metabolites.

Individual organic metabolites in the organeome or portion thereof may be identified by mass spectrometry, and/or by chromatography. The calculated synthesis or removal rates of the plurality of organic metabolites may be displayed.

In another aspect, the invention is drawn to methods of administering a diagnostic or therapeutic agent to the cell, tissue, or organism prior to administering the precursor. In one embodiment, the invention is drawn to a method of determining the effect of a diagnostic or therapeutic agent on a cell, tissue, or organism by determining the rates of synthesis or removal of a plurality of organic metabolites in the cell, tissue, or organism, administering an agent, and determining the rates of synthesis or removal on the plurality of organic metabolites in the cell, tissue or organism. By this means, drug discovery and development may be facilitated or achieved.

In another aspect, the invention is drawn to a method of determining the effects of one or more genes on the molecular flux rates of a plurality of organic metabolites in a cell, tissue, or organism by determining the molecular flux rates of a plurality of organic metabolites in a first population of one or more cells, tissues, or organisms having one or more genes; determining the molecular flux rates of the plurality of organic metabolites in a second population of one or more cells, tissues, or organisms that do not include the one or more genes, and comparing the molecular flux rates of said plurality of organic metabolites in the first and second populations.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a schematic diagram illustrating the organization of complex biological systems.

FIGURE 2 shows levels of functional genomics.

FIGURE 3 shows techniques for measuring dynamic proteomics.

FIGURE 4 depicts pathways of labeled hydrogen exchange from labeled water into selected free amino acids. Two nonessential amino acids (alanine, glycine) and an essential amino acid (leucine) are shown, by way of example. Alanine and glycine are presented in Figure 4A. Leucine is presented in Figure 4B. Abbreviations: TA, transaminase; PEP-CK, phosphoenol-pyruvate carboxykinase; TCAC, tricarboxylic acid cycle; STHM, serine tetrahydrofolate methyl transferase. Figure 4C depicts H_2^{18}O labeling of free amino acids for protein synthesis.

DETAILED DESCRIPTION OF THE INVENTION

The inventor has discovered a method of determining molecular flux rates (i.e., synthesis, breakdown and removal rates) of a plurality of proteins or a plurality of organic metabolites. First, an isotope-labeled precursor molecule is administered to a cell, tissue, or organism. The molecular flux rates of a plurality of proteins or a plurality of organic metabolites are then determined.

I. General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Introduction to Cell and*

Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); and *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). Furthermore, procedures employing commercially available assay kits and reagents will typically be used according to manufacturer-defined protocols unless otherwise noted.

II. Definitions

Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

"Molecular flux rates" refers to the rate of synthesis and/or breakdown of a protein and/or organic metabolite. "Molecular flux rates" also refers to a protein and/or organic metabolite's input into or removal from a pool of molecules, and is therefore synonymous with the flow into and out of said pool of molecules.

"Isotopologues" refer to isotopic homologues or molecular species that have identical elemental and chemical compositions but differ in isotopic content (*e.g.*, CH_3NH_2 vs. CH_3NHD in the example above). Isotopologues are defined by their isotopic composition, therefore each isotopologue has a unique exact mass but may not have a unique structure. An isotopologue usually includes of a family of isotopic isomers (isotopomers) which differ by the location of the isotopes on the molecule (*e.g.*, CH_3NHD and CH_2DNH_2 are the same isotopologue but are different isotopomers).

"Isotope-labeled water" includes water labeled with one or more specific heavy isotopes of either hydrogen or oxygen. Specific examples of isotope-labeled water include $^2\text{H}_2\text{O}$, $^3\text{H}_2\text{O}$, and H_2^{18}O .

"Protein Precursor" refers to any organic or inorganic molecule or component thereof, wherein one or more atoms of which are capable of being incorporated into protein molecules in cell, tissue, organism, or other biological system, through the biochemical processes of the cell, tissue, or organism. Examples of protein precursors include, but are not limited to, amino acids, H_2O , CO_2 , NH_3 , and HCO_3^- .

"Isotope Labeled protein precursor" refers to a protein precursor that contains an isotope of an element that differs from the most abundant isotope of the element present in nature, cells, tissue, or organisms. The isotope label may include specific heavy isotopes of elements present in biomolecules, such as ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S , ^{34}S , or may contain other isotopes of elements present in biomolecules such as ^3H , ^{14}C , ^{35}S , ^{125}I , ^{131}I . Isotope labeled protein precursors include, but are not limited to $^2\text{H}_2\text{O}$, $^{15}\text{NH}_3$, $^{13}\text{CO}_2$, $\text{H}^{13}\text{CO}_3^-$, ^2H -labeled amino acids, ^{13}C labeled amino acids, ^{15}N labeled amino acids, ^{18}O labeled amino acids, ^{34}S or ^{33}S labeled amino acids, $^3\text{H}_2\text{O}$ ^3H -labeled amino acids, and ^{14}C labeled amino acids.

"Isotope-labeled organic metabolite precursors" refer to an organic metabolite precursor that contains an isotope of an element that differs from the most abundant isotope of said element present in nature or cells, tissues, or organisms. Isotopic labels include specific heavy isotopes of elements, present in biomolecules, such as ^2H , ^{13}C , ^{15}N , ^{18}O , ^{35}S , ^{34}S , or may contain other isotopes of elements present in biomolecules, such as ^3H , ^{14}C , ^{35}S , ^{125}I , ^{131}I . Isotope labeled organic metabolite precursors include but are not limited to $^2\text{H}_2\text{O}$, $^{15}\text{NH}_3$, $^{13}\text{CO}_2$, H^{13}CO_3 , ^2H -labeled amino acids, ^{13}C -labeled amino acids, ^{15}N -labeled amino acids, ^{18}O -labeled amino acids, ^{33}S or ^{34}S -labeled amino acids, $^3\text{H}_2\text{O}$, ^3H -labeled amino acids, ^{14}C -labeled amino acids, $^{14}\text{CO}_2$, and H^{14}CO_2 .

"Partially purifying" refers to methods of removing one or more components of a mixture of other similar compounds. For example, "partially purifying a protein" refers to removing one or more proteins from a mixture of one or more proteins.

"Isolating" refers to separating one compound from a mixture of compounds. For example, "isolating a protein" refers to separating one specific protein from all other proteins in a mixture of one or more proteins.

A "biological sample" encompasses any sample obtained from a cell, tissue, or organism. The definition encompasses blood and other liquid samples of biological origin, that are accessible from an organism through sampling by minimally invasive or non-invasive approaches (*e.g.*, urine collection, blood drawing, needle aspiration, and other procedures involving minimal risk, discomfort or effort). The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or organic metabolites. The term "biological sample" also encompasses a clinical sample such as serum, plasma, other biological fluid, or tissue samples, and also includes cells in culture, cell supernatants and cell lysates.

"Biological fluid" refers, but is not limited to, urine, blood, interstitial fluid, edema fluid, saliva, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, empyema or other infected fluid, cerebrospinal fluid, sweat, pulmonary secretions (sputum), seminal fluid, feces, bile, intestinal secretions, or other biological fluid.

"Exact mass" refers to mass calculated by summing the exact masses of all the isotopes in the formula of a molecule (e.g. 32.04847 for CH₃NHD).

"Nominal mass" refers to the integer mass obtained by rounding the exact mass of a molecule.

"Mass isotopomer" refers to family of isotopic isomers that is grouped on the basis of nominal mass rather than isotopic composition. A mass isotopomer may comprise molecules of different isotopic compositions, unlike an isotopologue (e.g. CH₃NHD, ¹³CH₃NH₂, CH₃¹⁵NH₂ are part of the same mass isotopomer but are different isotopologues). In operational terms, a mass isotopomer is a family of isotopologues that are not resolved by a mass spectrometer. For quadrupole mass spectrometers, this typically means that mass isotopomers are families of isotopologues that share a nominal mass. Thus, the isotopologues CH₃NH₂ and CH₃NHD differ in nominal mass and are distinguished as being different mass isotopomers, but the isotopologues CH₃NHD, CH₂DNH₂, ¹³CH₃NH₂, and CH₃¹⁵NH₂ are all of the same nominal mass and hence are the same mass isotopomers. Each mass isotopomer is therefore typically composed of more than one isotopologue and has more than one exact mass. The distinction between isotopologues and mass isotopomers is useful in practice because all individual isotopologues are not resolved using quadrupole mass spectrometers and may not be resolved even using mass spectrometers that produce higher mass resolution, so that calculations from mass spectrometric data must be performed on the abundances of mass isotopomers rather than isotopologues. The mass isotopomer lowest in mass is represented as M₀; for most organic molecules, this is the species containing all ¹²C, ¹H, ¹⁶O, ¹⁴N, etc. Other mass isotopomers are distinguished by their mass differences from M₀ (M₁, M₂, etc.). For a given mass isotopomer, the location or position of isotopes within the molecule is not specified and may vary (i.e. "positional isotopomers" are not distinguished).

"Mass isotopomer envelope" refers to the set of mass isotopomers associated with a molecule or ion fragment.

"Mass isotopomer pattern" refers to a histogram of the abundances of the mass isotopomers of a molecule. Traditionally, the pattern is presented as percent relative abundances where all of the abundances are normalized to that of the most abundant mass isotopomer; the most abundant isotopomer is said to be 100%. The preferred form for applications involving probability analysis, such as mass isotopomer distribution analysis (MIDA), however, is proportion or fractional abundance, where the fraction that each species contributes to the total abundance is used. The term "isotope pattern" may be used synonymously with the term "mass isotopomer pattern."

"Monoisotopic mass" refers to the exact mass of the molecular species that contains all ^1H , ^{12}C , ^{14}N , ^{16}O , ^{32}S , etc. For isotopologues composed of C, H, N, O, P, S, F, Cl, Br, and I, the isotopic composition of the isotopologue with the lowest mass is unique and unambiguous because the most abundant isotopes of these elements are also the lowest in mass. The monoisotopic mass is abbreviated as m_0 and the masses of other mass isotopomers are identified by their mass differences from m_0 (m_1 , m_2 , etc.).

"Isotopically perturbed" refers to the state of an element or molecule that results from the explicit incorporation of an element or molecule with a distribution of isotopes that differs from the distribution found in nature, whether a naturally less abundant isotope is present in excess (enriched) or in deficit (depleted).

"Monomer" refers to a chemical unit that combines during the synthesis of a polymer and which is present two or more times in the polymer.

"Polymer" refers to a molecule synthesized from and containing two or more repeats of a monomer.

"Protein" refers to a polymer of amino acids. As used herein, a "protein" may be to a long amino acid polymers as well as short polymers such as peptides.

"Proteome" refers to the complement of proteins expressed by a cell, tissue or organism under a specific set of conditions.

"Static proteomics" refers to current mass spectrometric techniques well-known in the art for characterizing the protein complement expressed in a cell, tissue or

organism by their concentrations or levels but not as the rates of synthesis or breakdown (fluxes) of these proteins (to be contrasted with Dynamic Proteomics).

"Dynamic proteomics" refers to mass spectrometric techniques for characterizing the rates of synthesis and/or breakdown (fluxes) of the proteins in a proteome.

"Organic metabolite" refers to any organic molecule involved in metabolism in a cell, tissue, or organism. Organic metabolites may include, but are not limited to, amino acids, sugars, sugar alcohols, organic acids, sterols, and nucleotide bases.

"Organeome" refers to the population of organic molecules present in a cell, tissue or organism. "Organeome" may also refer to the population of organic molecules present in a cell, tissue or organism under a specific set of conditions.

"Static organeomics" refers to current mass spectrometric techniques well known in the art for characterizing the complement of organic molecules or metabolites present in a cell, tissue or organism by their concentrations or levels, but not by the rates of synthesis or breakdown of these organic molecules or metabolites.

"Dynamic organeomics" refers mass spectrometric techniques for characterizing the rates of synthesis and/or breakdown of the organic molecules or metabolites in an organeome.

"Organic metabolite precursor" refers to an organic or inorganic molecule capable of entering into cellular pools of organic metabolites either directly or by prior transformation.

III. Methods of the Invention

The present invention is directed to methods of determining the molecular flux rates of a plurality of proteins in all or a portion of the proteome of a cell, tissue or organism. First, one or more isotope-labeled protein precursors are administered to a cell, tissue or organism for a period of time sufficient to be incorporated into a plurality of proteins in the proteome or portion thereof. The proteome or portion thereof is obtained from the cell, tissue, or organism, and a plurality of individual proteins are identified by mass spectrometry. The relative and absolute mass isotopomer abundances of the ions within the isotopomeric envelope corresponding to each

identified protein or peptide are quantified by mass spectrometry, and the molecular flux rates of each identified protein or peptide to determine the molecular flux rates of said plurality of proteins.

The same methodology may be applied to determine the molecular flux rates of a plurality of organic metabolites in all or a portion of the organeome.

The organization of complex biological systems is illustrated in **Figure 1**. The levels of functional genomics are illustrated in **Figure 2**. The present invention is directed to methods of measuring, analyzing, quantitating, qualifying and interpreting dynamic proteomic measurements and dynamic organeomic measurements. (See **Figure 3**).

Administering Isotope-Labeled Precursor(s)

As a first step in the methods of the invention, isotope-labeled precursors are administered.

A. Administering an Isotope-Labeled Precursor Molecule

1. Labeled precursor molecules

a. Isotope labels

The first step in measuring molecular flux rates involves administering an isotope-labeled precursor molecule to a cell, tissue, or organism. The isotope labeled precursor molecule may be a stable isotope or radioisotope. Isotope labels that can be used include, but are not limited to, ^2H , ^{13}C , ^{15}N , ^{18}O , ^3H , ^{14}C , ^{35}S , ^{32}P , ^{125}I , ^{131}I , or other isotopes of elements present in organic systems.

In one embodiment, the isotope label is ^2H .

b. Precursor Molecules

The precursor molecule may be any molecule having an isotope label that is incorporated into a protein or organic metabolite. Isotope labels may be used to modify all precursor molecules disclosed herein to form isotope-labeled precursor molecules.

The entire precursor molecule may be incorporated into one or more proteins and/or organic metabolites. Alternatively, a portion of the precursor molecule may be incorporated into one or more proteins and/or organic metabolite.

Precursor molecules may include, but not limited to, CO₂, NH₃, glucose, lactate, H₂O, acetate, and fatty acids.

i. *Protein Precursors*

A protein precursor molecule may be any protein precursor molecule known in the art. These precursor molecules may be CO₂, NH₃, glucose, lactate, H₂O, acetate, and fatty acids.

Precursor molecules of proteins may also include one or more amino acids. The precursor may be any amino acid. The precursor molecule may be a singly or multiply deuterated amino acid. For example, the precursor molecule may be one or more of ¹³C-lysine, ¹⁵N-histidine, ¹³C-serine, ¹³C-glycine, ²H-leucine, ¹⁵N-glycine, ¹³C-leucine, ²H₅-histidine, and any deuterated amino acid. Labeled amino acids may be administered, for example, undiluted or diluted with non-labeled amino acids. All isotope labeled precursors may be purchased commercially, for example, from Cambridge Isotope Labs (Andover, MA).

Protein precursor molecules may also include any precursor for post-translational or pre-translationally modified amino acids. These precursors include but are not limited to precursors of methylation such as glycine, serine or H₂O; precursors of hydroxylation, such as H₂O or O₂; precursors of phosphorylation, such as phosphate, H₂O or O₂; precursors of prenylation, such as fatty acids, acetate, H₂O, ethanol, ketone bodies, glucose, or fructose; precursors of carboxylation, such as CO₂, O₂, H₂O, or glucose; precursors of acetylation, such as acetate, ethanol, glucose, fructose, lactate, alanine, H₂O, CO₂, or O₂; and other post-translational modifications known in the art.

The degree of labeling present in free amino acids may be determined experimentally, or may be assumed based on the number of labeling sites in an amino acid. For example, when using hydrogen isotopes as a label, the labeling present in C-H bonds of free amino acid or, more specifically, in tRNA-amino acids, during exposure to ²H₂O in body water may be identified. The total number of C-H bonds in each non essential amino acid is known - e.g. 4 in alanine, 2 in glycine, etc.

The precursor molecule for proteins may be water. The hydrogen atoms on C-H bonds are the hydrogen atoms on amino acids that are useful for measuring protein

synthesis from $^2\text{H}_2\text{O}$ since the O-H and N-H bonds of proteins are labile in aqueous solution. As such, the exchange of ^2H -label from $^2\text{H}_2\text{O}$ into O-H or N-H bonds occurs without the synthesis of proteins from free amino acids as described above. C-H bonds undergo incorporation from H_2O into free amino acids during specific enzyme-catalyzed intermediary metabolic reactions (Figure 4). The presence of ^2H -label in C-H bonds of protein-bound amino acids after $^2\text{H}_2\text{O}$ administration therefore means that the protein was assembled from amino acids that were in the free form during the period of $^2\text{H}_2\text{O}$ exposure - i.e. that the protein is newly synthesized. Analytically, the amino acid derivative used must contain all the C-H bonds but must remove all potentially contaminating N-H and O-H bonds.

Hydrogen atoms from body water may be incorporated into free amino acids. ^2H or ^3H from labeled water can enter into free amino acids in the cell through the reactions of intermediary metabolism, but ^2H or ^3H cannot enter into amino acids that are present in peptide bonds or that are bound to transfer RNA. Free essential amino acids may incorporate a single hydrogen atom from body water into the α -carbon C-H bond, through rapidly reversible transamination reactions (Figure 4). Free non-essential amino acids contain a larger number of metabolically exchangeable C-H bonds, of course, and are therefore expected to exhibit higher isotopic enrichment values per molecule from $^2\text{H}_2\text{O}$ in newly synthesized proteins (Figures 4A-B).

One of skill in the art will recognize that labeled hydrogen atoms from body water may be incorporated into other amino acids via other biochemical pathways. For example, it is known in the art that hydrogen atoms from water may be incorporated into glutamate via synthesis of the precursor α -ketoglutarate in the citric acid cycle. Glutamate, in turn, is known to be the biochemical precursor for glutamine, proline, and arginine. By way of another example, hydrogen atoms from body water may be incorporated into post-translationally modified amino acids, such as the methyl group in 3-methyl-histidine, the hydroxyl group in hydroxyproline or hydroxylysine, and others. Other amino acids synthesis pathways are known to those of skill in the art.

Oxygen atoms (H_2^{18}O) may also be incorporated into amino acids through enzyme-catalyzed reactions. For example, oxygen exchange into the carboxylic acid moiety of

amino acids may occur during enzyme catalyzed reactions. Incorporation of labeled oxygen into amino acids is known to one of skill in the art as illustrated in Figure 4C. Oxygen atoms may also be incorporated into amino acids from $^{18}\text{O}_2$ through enzyme catalyzed reactions (including hydroxyproline, hydroxylysine or other post-translationally modified amino acids).

Hydrogen and oxygen labels from labeled water may also be incorporated into amino acids through post-translational modifications. In one embodiment, the post-translational modification may already include labeled hydrogen or oxygen through biosynthetic pathways prior to post-translational modification. In another embodiment, the post-translational modification may incorporate labeled hydrogen, oxygen, carbon, or nitrogen from metabolic derivatives involved in the free exchange labeled hydrogens from body water, either before or after post-translational modification step (e.g. methylation, hydroxylation, phosphorylation, prenylation, sulfation, carboxylation, acetylation or other known post-translational modifications).

Protein precursors for that are suitable for administration into a subject include, but are not limited to H_2O , CO_2 , NH_3 and HCO_3^- , in addition to the standard amino acids found in proteins.

ii. Precursors of Organic Metabolites

Precursors of organic metabolites may be any precursor molecule capable of entering into the organic metabolite pathway. Organic metabolites and organic metabolite precursors include H_2O , CO_2 , NH_3 , HCO_3^- , amino acids, monosaccharides, carbohydrates, lipids, fatty acids, nucleic acids, glycolytic intermediates, acetic acid, and tricarboxylic acid cycle intermediates.

Organic metabolite precursors may also be administered directly. Mass isotopes may be useful in mass isotope labeling of protein or organic metabolite precursors include, but are not limited to ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S and ^{34}S . It is often desirable, in order to avoid metabolic loss of isotope labels, that the isotope-labeled atom(s) be relatively non-labile or at least behave in a predictable manner within the subject. By administering the isotope-labeled precursors to the biosynthetic pool, the

isotope-labeled precursors can become directly incorporated into organic metabolites formed in the pool.

iii. Water as a Precursor Molecule

Water is a precursor of proteins and many organic metabolites. As such, labeled water may serve as a precursor in the methods taught herein.

Labeled water may be readily obtained commercially. For example, $^2\text{H}_2\text{O}$ may be purchased from Cambridge Isotope Labs (Andover, MA), and $^3\text{H}_2\text{O}$ may be purchased, e.g., from New England Nuclear, Inc. In general, $^2\text{H}_2\text{O}$ is non-radioactive and thus, presents fewer toxicity concerns than radioactive $^3\text{H}_2\text{O}$. $^2\text{H}_2\text{O}$ may be administered, for example, as a percent of total body water, e.g., 1% of total body water consumed (e.g., for 3 litres water consumed per day, 30 microliters $^2\text{H}_2\text{O}$ is consumed). If $^3\text{H}_2\text{O}$ is utilized, then a non-toxic amount, which is readily determined by those of skill in the art, is administered.

Relatively high body water enrichments of $^2\text{H}_2\text{O}$ (e.g., 1-10% of the total body water is labeled) may be achieved relatively inexpensively using the techniques of the invention. This water enrichment is relatively constant and stable as these levels are maintained for weeks or months in humans and in experimental animals without any evidence of toxicity. This finding in a large number of human subjects (> 100 people) is contrary to previous concerns about vestibular toxicities at high doses of $^2\text{H}_2\text{O}$. The Applicant has discovered that as long as rapid changes in body water enrichment are prevented (e.g., by initial administration in small, divided doses), high body water enrichments of $^2\text{H}_2\text{O}$ can be maintained with no toxicities. For example, the low expense of commercially available $^2\text{H}_2\text{O}$ allows long-term maintenance of enrichments in the 1-5% range at relatively low expense (e.g., calculations reveal a lower cost for 2 months labeling at 2% $^2\text{H}_2\text{O}$ enrichment, and thus 7-8% enrichment in the alanine precursor pool (Figure 4A-B), than for 12 hours labeling of ^2H -leucine at 10% free leucine enrichment, and thus 7-8% enrichment in leucine precursor pool for that period).

Relatively high and relatively constant body water enrichments for administration of H_2^{18}O may also be accomplished, since the ^{18}O isotope is not toxic, and does not present a significant health risk as a result (Figure 4C).

iv. Modes of Administering Precursors of Proteins and Organic Metabolites

Modes of administering the one or more isotope-labeled precursors may vary, depending upon the absorptive properties of the isotope-labeled precursor and the specific biosynthetic pool into which each compound is targeted. Precursors may be administered to organisms, plants and animals including humans directly for *in vivo* analysis. In addition, precursors may be administered *in vitro* to living cells. Specific types of living cells include hepatocytes, adipocytes, myocytes, fibroblasts, neurons, pancreatic β -cells, intestinal epithelial cells, leukocytes, lymphocytes, erythrocytes, microbial cells and any other cell-type that can be maintained alive and functional *in vitro*.

Generally, an appropriate mode of administration is one that produces a steady state level of precursor within the biosynthetic pool and/or in a reservoir supplying such a pool for at least a transient period of time. Intravenous or oral routes of administration are commonly used to administer such precursors to organisms, including humans. Other routes of administration, such as subcutaneous or intramuscular administration, optionally when used in conjunction with slow release precursor compositions, are also appropriate. Compositions for injection are generally prepared in sterile pharmaceutical excipients.

B. Obtaining a plurality of proteins or organic metabolites

In practicing the method of the invention, in one aspect, proteins and organic metabolites are obtained from a cell, tissue, or organism according to the methods known in the art. The methods may be specific to the proteins or organic metabolites of interest. Proteins and organic metabolites of interest may be isolated from a biological sample.

A plurality of proteins or a plurality of organic metabolites may be acquired from the cell, tissue, or organism. The one or more biological samples may be obtained, for example, by blood draw, urine collection, biopsy, or other methods known in the art.

The one or more biological sample may be one or more biological fluids. The protein or organic metabolite may also be obtained from specific organs or tissues, such as muscle, liver, adrenal tissue, prostate tissue, endometrial tissue, blood, skin, and breast tissue. Proteins or organic metabolites may be obtained from a specific group of cells, such as tumor cells or fibroblast cells. Proteins or organic metabolites also may be obtained, and optionally partially purified or isolated, from the biological sample using standard biochemical methods known in the art.

The frequency of biological sampling can vary depending on different factors. Such factors include, but are not limited to, the nature of the proteins or organic metabolites, ease and safety of sampling, synthesis and breakdown/removal rates of the proteins or organic metabolites from which it was derived, and the half-life of a therapeutic agent or biological agent.

The proteins or organic metabolites may also be purified partially, or optionally, isolated, by conventional purification methods including high pressure liquid chromatography (HPLC), fast performance liquid chromatography (FPLC), chemical extraction, thin layer chromatography, gas chromatography, gel electrophoresis, and/or other separation methods known to those skilled in the art.

In another embodiment, the proteins or organic metabolites may be hydrolyzed or otherwise degraded to form smaller molecules. Hydrolysis methods include any method known in the art, including, but not limited to, chemical hydrolysis (such as acid hydrolysis) and biochemical hydrolysis (such as peptidase degradation). Hydrolysis or degradation may be conducted either before or after purification and/or isolation of the proteins or organic metabolites. The proteins or organic metabolites also may be partially purified, or optionally, isolated, by conventional purification methods including high performance liquid chromatography (HPLC), fast performance liquid chromatography (FPLC), gas chromatography, gel electrophoresis, and/or any other methods of separating chemical and/or biochemical compounds known to those skilled in the art.

C. Analysis

Presently available technologies (static proteomics) used to profile differences in expressed proteins measure only protein levels (concentrations) in a cell and do so at one point in time. Approaches include Global Proteome Mapping (using 2D technology and Mass Spectrometry), Differential Expression Analysis (using 2D/DIGE technology and Mass Spectrometry), and Expression Analysis (2D/DIGE, Mass Spectrometry and LC-based, or other novel technology). While RNA and protein expression "chips" can be used to rapidly detect biologically mediated resistance to a therapeutic agent in a variety of disease states, they fail to determine the rate of change of gene expression or protein turnover. The methods of the present invention allow determination of the *rates* of gene expression and molecular flux rates of a plurality of proteins, as well as the molecular flux rates of a plurality of organic metabolites, and their changes over time.

Mass Spectrometry

Isotopic enrichment in proteins and organic metabolites can be determined by various methods such as mass spectrometry, including but not limited to gas chromatography-mass spectrometry (GC-MS), isotope-ratio mass spectrometry, GC-isotope ratio-combustion-MS, GC-isotope ratio-pyrolysis-MS, liquid chromatography-MS, electrospray ionization-MS, matrix assisted laser desorption-time of flight-MS, Fourier-transform-ion-cyclotron-resonance-MS, and cyclodial-MS.

Mass spectrometers convert molecules such as proteins and organic metabolites into rapidly moving gaseous ions and separate them on the basis of their mass-to-charge ratios. The distributions of isotopes or isotopologues of ions, or ion fragments, may thus be used to measure the isotopic enrichment in a plurality of proteins or organic metabolites.

Generally, mass spectrometers include an ionization means and a mass analyzer. A number of different types of mass analyzers are known in the art. These include, but are not limited to, magnetic sector analyzers, electrospray ionization, quadrupoles, ion traps, time of flight mass analyzers, and Fourier transform analyzers.

Mass spectrometers may also include a number of different ionization methods. These include, but are not limited to, gas phase ionization sources such as electron impact, chemical ionization, and field ionization, as well as desorption sources, such as field desorption, fast atom bombardment, matrix assisted laser desorption/ionization, and surface enhanced laser desorption/ionization.

In addition, two or more mass analyzers may be coupled (MS/MS) first to separate precursor ions, then to separate and measure gas phase fragment ions. These instruments generate an initial series of ionic fragments of a protein, and then generate secondary fragments of the initial ions. The resulting overlapping sequences allows complete sequencing of the protein, by piecing together overlaying "pieces of the puzzle", based on a single mass spectrometric analysis within a few minutes (plus computer analysis time).

The MS/MS peptide fragmentation patterns and peptide exact molecular mass determinations generated by protein mass spectrometry provide unique information regarding the amino acid sequence of proteins and find use in the present invention. An unknown protein can be sequenced and identified in minutes, by a single mass spectrometric analytic run. The library of peptide sequences and protein fragmentation patterns that is now available provides the opportunity to identify components of complex proteome mixtures with near certainty.

Different ionization methods are also known in the art. One key advance has been the development of techniques for ionization of large, non-volatile macromolecules including proteins and polynucleotides. Techniques of this type have included electrospray ionization (ESI) and matrix assisted laser desorption (MALDI). These have allowed MS to be applied in combination with powerful sample separation introduction techniques, such as liquid chromatography and capillary zone electrophoresis.

In addition, mass spectrometers may be coupled to separation means such as gas chromatography (GC) and high performance liquid chromatography (HPLC). In gas-chromatography mass-spectrometry (GC/MS), capillary columns from a gas chromatograph are coupled directly to the mass spectrometer, optionally using a jet separator. In such an application, the gas chromatography (GC) column separates

sample components from the sample gas mixture and the separated components are ionized and chemically analyzed in the mass spectrometer.

When GC/MS (or other mass spectrometric modalities that analyze ions of proteins and organic metabolites, rather than small inorganic gases) is used to measure mass isotopomer abundances of organic molecules, hydrogen-labeled isotope incorporation from isotope-labeled water is amplified 3 to 7-fold, depending on the number of hydrogen atoms incorporated into the organic molecule from isotope-labeled water in vivo.

In general, in order to determine a baseline mass isotopomer frequency distribution for the protein, such a sample is taken before infusion of an isotopically labeled precursor. Such a measurement is one means of establishing in the cell, tissue or organism, the naturally occurring frequency of mass isotopomers of the protein. When a cell, tissue or organism is part of a population of subjects having similar environmental histories, a population isotopomer frequency distribution may be used for such a background measurement. Additionally, such a baseline isotopomer frequency distribution may be estimated, using known average natural abundances of isotopes. For example, in nature, the natural abundance of ^{13}C present in organic carbon is 1.11%. Methods of determining such isotopomer frequency distributions are discussed below. Typically, samples of the protein are taken prior to and following administration of an isotopically labeled precursor to the subject and analyzed for isotopomer frequency as described below. Similar considerations apply to the isolation of organic molecules for Dynamic Organomics.

Thus, a single analysis of even an enormously complex mixture of proteins (that has been subjected to proteolytic cleavage or analyzed directly) can uniquely identify peptides representing thousands of expressed proteins.

Proteins may also be detected using protein chips. Several commercial "protein chip" equivalents are now marketed, using mass spectrometry (e.g. Ciphergen Biosystems). The efficiency of peptide sequence determination by mass analysis, combined with powerful ion fragmentation technology (MS/MS instruments) and/or peptide generating biochemical methods (e.g. proteolysis), improvements in sample

introduction methods (HPLC, surface desorption, etc.), improved capacity for ionization of even the largest macromolecules (ESI, MALDI/SELDI) and rapid computerized handling of large data sets and comparison to peptide/protein reference libraries, have made mass spectrometry a general and powerful tool for automated, large-scale, high-throughput static proteomics.

Identification of a Plurality of Proteins or Organic Molecules

The plurality of proteins or organic molecules is analyzed by mass spectrometry, using standard methods well known in the art. The following references describe the application of static mass spectrometric techniques to protein identification, with respect to proteome analysis in particular: Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlett DR, Aebersold R, Hood L "Integrated genomic and proteomic analyses of a systemically perturbed metabolic network." *Science*. 2001 May 4; 292 (5518): 929-34; Gygi SP, Aebersold R. "Mass spectrometry and proteomics." *Curr Opin Chem Biol*. 2000 Oct; 4 (5): 489-94; Gygi SP, Rist B, Aebersold R "Measuring gene expression by quantitative proteome analysis" *Curr Opin Biotechnol*. 2000 Aug; 11 (4): 396-401; Goodlett DR, Bruce JE, Anderson GA, Rist B, Pasatolic L, Fiehn O, Smith RD, Aebersold R. "Protein Identification with a single accurate mass of a cysteine-containing peptide and constrained database searching" *Anal Chem*. 2000 Mar 15; 72 (6): 1112-8; and Goodlett DR, Aebersold R, Watts JD "Quantitative in vitro kinase reaction as a guide for phosphoprotein analysis by mass spectrometry" *Rapid Commun Mass Spectrom*. 2000; 14 (5): 344-8; Zhou, H. et al (Apr 2001) *Nature Biotechnol*. 19: 375-378.

Protein biochips, also known as protein arrays or antibody arrays, are used to identify proteins. These biochips hold the potential to measure protein-protein interactions, protein-small molecule interactions, and enzyme-substrate reactions. They can also distinguish the proteins of a healthy cell from those of a diseased cell. Protein biochips draw on the DNA chip technology developed for genomics and are also able to analyze thousands of samples simultaneously. While the human genome may contain

100,000 genes, post-translational modifications and RNA splicing events result in far greater than 100,000 proteins.

Some biochips incorporate a type of mass spectrometry called surface enhanced laser desorption/ionization [SELDI], and biochip technology in a single, integrated platform, allowing the proteins to be captured, separated, and quantitatively analyzed directly on the chip. The chips are read directly by the SELDI process without radioactive or fluorescent labels or genetically engineered tags.

While these techniques are useful, they merely provide a static assessment of the proteome, not a dynamic assessment of the proteome. The methods herein provide such a dynamic assessment.

Identification of a plurality of organic metabolites

The sample containing organic metabolites is analyzed by mass spectrometry, using standard methods well known in the art. The following reference describes the application of static mass spectrometric techniques to metabolite identification, with respect to organic metabolite identification: Wolfe, R. R. Radioactive and Stable Isotope Tracers In Biomedicine: Principles and Practice of Kinetic Analysis. John Wiley & Sons; (March 1992).

The pattern of intermediary metabolites and their concentrations in living systems represents a still-higher level of biochemical phenotype. Myriad organic molecules are present in living systems and serve as substrates for the enzymes that control flows through functional biochemical pathways. A plurality of organic metabolites may be most effectively accomplished by use of ESI-MS/MS or GC/MS approaches.

Spots of blood or urine are introduced into an MS device and characterized by chromatographic behavior and mass spectrum.

This approach has been used for diagnostic screening of urine samples for a wide range of organic metabolites to detect inborn errors of metabolism in children. Plant biochemists have also reported some metabolic profiling work concerning the functional biochemistry of plants.

Technically, it is easier to isolate organic metabolites than proteins using traditional mass spectrometers (e.g. GC/MS instruments) because organic metabolites are generally of smaller size and greater volatility than proteins. Other features of organeomics strongly support the potential applicability of mass spectrometry (e.g. a very large number of analytes that need to be measured concurrently; requirement to measure concentrations; the need for automation and complex data handling; and the need for comparison to informatics libraries).

Measuring Relative and Absolute Mass Isotopomer Abundances

Measured mass spectral peak heights, or alternatively, the areas under the peaks, may be expressed as ratios toward the parent (zero mass isotope) isotopomer. It is appreciated that any calculation means which provide relative and absolute values for the abundances of isotopomers in a sample may be used in describing such data, for the purposes of the invention.

Calculating Labeled: Unlabeled Proportion of Proteins and Organic Metabolites

The proportion of labeled and unlabeled proteins or organic metabolites is then calculated. The practitioner first determines measured excess molar ratios for isolated isotopomer species of a molecule. The practitioner then compares measured internal pattern of excess ratios to the theoretical patterns. Such theoretical patterns can be calculated using the binomial or multinomial distribution relationships as described in U.S. Patents Numbers 5,338,686; 5,910,403; and 6,010,846 which are hereby incorporated by reference in their entirety. The calculations may include Mass Isotopomer Distribution Analysis (MIDA). Variations of Mass Isotopomer Distribution Analysis (MIDA) combinatorial algorithm are discussed in a number of different sources known to one skilled in the art. The method is further discussed by Hellerstein and Neese (1999), as well as Chinkes, et al. (1996), and Kelleher and Masterson (1992), and U.S. Patent Application No. 10/279,399, all of which are hereby incorporated by reference in their entirety.

In addition to the above-cited references, calculation software implementing the method is publicly available from Professor Marc Hellerstein, University of California, Berkeley.

The comparison of excess molar ratios to the theoretical patterns can be carried out using a table generated for a protein of interest, or graphically, using determined relationships. From these comparisons, a value, such as the value p , is determined, which describes the probability of mass isotopic enrichment of a subunit in a precursor subunit pool. This enrichment is then used to determine a value, such as the value A_x^* , which describes the enrichment of newly synthesized proteins for each mass isotopomer, to reveal the isotopomer excess ratio which would be expected to be present, if all isotopomers were newly synthesized.

Fractional abundances are then calculated. Fractional abundances of individual isotopes (for elements) or mass isotopomers (for molecules) are the fraction of the total abundance represented by that particular isotope or mass isotopomer. This is distinguished from relative abundance, wherein the most abundant species is given the value 100 and all other species are normalized relative to 100 and expressed as percent relative abundance. For a mass isotopomer M_x

$$\text{Fractional abundance of } M_x = A_x = \frac{\text{Abundance } M_x}{\sum_{i=0}^n \text{Abundance } M_i}, \text{ where } 0 \text{ to } n \text{ is the range}$$

of nominal masses relative to the lowest mass (M_0) mass isotopomer in which abundances occur.

Δ Fractional abundance (enrichment or depletion) =

$$(A_x)_e - (A_x)_b = \left(\frac{\text{Abundance } M_x}{\sum_{i=0}^n \text{Abundance } M_i} \right)_e - \left(\frac{\text{Abundance } M_x}{\sum_{i=0}^n \text{Abundance } M_i} \right)_b,$$

where subscript e refers to enriched and b refers to baseline or natural abundance.

In order to determine the fraction of polymers that were actually newly synthesized during a period of precursor administration, the measured excess molar

ratio (EM_x) is compared to the calculated enrichment value, A_x^* , which describes the enrichment of newly synthesized biopolymers for each mass isotopomer, to reveal the isotopomer excess ratio which would be expected to be present, if all isotopomers were newly synthesized.

Calculating Molecular Flux Rates

The method of determining rate of synthesis includes calculating the proportion of mass isotopically labeled subunit present in the protein precursor pool, and using this proportion to calculate an expected frequency of a protein containing at least one mass isotopically labeled subunit. This expected frequency is then compared to the actual, experimentally determined protein isotopomer frequency. From these values, the proportion of protein which is synthesized from added isotopically labeled precursors during a selected incorporation period can be determined. Thus, the rate of synthesis during such a time period is also determined.

A precursor-product relationship is then applied. For the continuous labeling method, the isotopic enrichment is compared to asymptotic (*i.e.*, maximal possible) enrichment and kinetic parameters (*e.g.*, synthesis rates) are calculated from precursor-product equations. The fractional synthesis rate (k_s) may be determined by applying the continuous labeling, precursor-product formula:

$$k_s = [-\ln(1-f)]/t,$$

where f = fractional synthesis = product enrichment/asymptotic precursor/enrichment

and t = time of label administration of contacting in the system studied.

For the discontinuous labeling method, the rate of decline in isotope enrichment is calculated and the kinetic parameters of proteins are calculated from exponential decay equations. In practicing the method, biopolymers are enriched in mass isotopomers, preferably containing multiple mass isotopically labeled precursors. These higher mass isotopomers of the proteins, *e.g.*, proteins containing 3 or 4 mass isotopically labeled precursors, are formed in negligible amounts in the absence of

exogenous precursor, due to the relatively low abundance of natural mass isotopically labeled precursor, but are formed in significant amounts during the period of protein precursor incorporation. The proteins taken from the cell, tissue, or organism at the sequential time points are analyzed by mass spectrometry, to determine the relative frequencies of a high mass protein isotopomer. Since the high mass isotopomer is synthesized almost exclusively before the first time point, its decay between the two time points provides a direct measure of the rate of decay of the protein.

Preferably, the first time point is at least 2-3 hours after administration of precursor has ceased, depending on mode of administration, to ensure that the proportion of mass isotopically labeled subunit has decayed substantially from its highest level following precursor administration. In one embodiment, the following time points are typically 1-4 hours after the first time point, but this timing will depend upon the replacement rate of the biopolymer pool.

The rate of decay of the protein is determined from the decay curve for the three-isotope protein. In the present case, where the decay curve is defined by several time points, the decay kinetics can be determined by fitting the curve to an exponential decay curve, and from this, determining a decay constant.

Breakdown rate constants (k_d) may be calculated based on an exponential or other kinetic decay curve:

$$k_d = [-\ln f]/t.$$

While the invention has been described with respect to specific mass isotopes and proteins, it will be appreciated how the method can be used to determine subunit pool composition, and rates of synthesis and decay for substantially any biopolymer which is formed from two or more identical subunits which can be mass isotopically labeled. Similar considerations apply for organic metabolites.

Uses of the Techniques of the Present Invention

Examples of medically relevant metabolic determinations which can be made, using the methods of the invention include: i) molecular flux rates of proteins involved

in fat or cholesterol synthesis in a cell, tissue, or organism, to determine nutritional effects and/or the effects of drug treatment; ii) molecular flux rates of plasma proteins, as may occur in certain disease states before, during and after treatment with various drugs; iii) muscle protein dynamics, to determine effects of such determinants as exercise, hormones, drug treatment, age and disease on synthesis and breakdown of muscle protein; iv) rates of protein synthesis, including viral replication rates *in vivo*, for assessment of antiviral drugs on such rates *in vivo*, and rates of protein synthesis and degradation in a tumor, to determine the efficacy of chemotherapy; v) study of changes in gluconeogenesis, as may be affected by diseases such as diabetes, cancer and hypoglycemia. Normal tissue and diseased tissue can often be distinguished by the types of active genes and their expression levels; furthermore, the progression of disease can be determined by knowing the rate of change of protein synthesis or breakdown. Such testing can be performed *in vivo* directly on human subjects or *ex vivo* using cell cultures. Cell cultures may include animal cells such as human cells, plant cells, microbial cells including fungi, yeast and bacteria.

Altered expression patterns of oncogenes and tumor suppressor genes, for example, are reflected in changes in the synthesis and/or breakdown rates of the proteins that they code, and can effect dramatic changes in the expression profiles of numerous other genes. Different protein turnover or organic metabolite fluxes can serve as markers of the transformed state and are, therefore, of potential value in the diagnosis and classification of tumors. Differences in gene expression, which are not the cause but rather the effect of transformation, may be used as markers for the tumor stage. Thus, the assessment of the protein kinetic consequences of known tumor-associated genes has the potential to provide meaningful information with respect to tumor type and stage, treatment methods, and prognosis. Furthermore, new tumor-associated genes may be identified by systemically correlating their functional consequences on protein or organic metabolite fluxes with their level of gene expression in tumor specimens and control tissue. Genes whose expression is increased or reduced in tumors relative to normal cells, in association with altered fluxes of proteins or organic metabolites, are candidates for classification as oncogenes, tumor

suppressor genes or genes encoding apoptosis-inducing products. Generally, the underlying premise is that the profiles of dynamic protein or organic metabolite fluxes may provide more specific, direct and accurate markers of gene function than cruder, less biochemical and less systematic markers of phenotype can provide. By this means, the physiological function or malfunction of the gene product in the organism can be established – i.e. true “functional genomics” become possible.

These practical applications can help physicians reduce health care costs, achieve rapid therapeutic benefits, limit administration of ineffective yet toxic drugs, and monitor changes in (e.g., decreases in) pathogenic resistance.

Kits

The invention provides kits for measuring and comparing molecular flux rates in vivo. The kits may include isotope-labeled precursor molecules, and in preferred embodiments, chemical compounds known in the art for separating, purifying, or isolating proteins, and/or chemicals necessary to obtain a tissue sample, automated calculation software for combinatorial analysis, and instructions for use of the kit.

Other kit components, such as tools for administration of water (e.g., measuring cup, needles, syringes, pipettes, IV tubing), may optionally be provided in the kit. Similarly, instruments for obtaining samples from the cell, tissue, or organism (e.g., specimen cups, needles, syringes, and tissue sampling devices) may also be optionally provided.

ADVANTAGES OF THE PRESENT INVENTION

The field of the current invention relates to the measurement of Dynamic Proteomics and Dynamic Organeomics – the kinetics (i.e., the molecular flux rates – synthesis and breakdown rates; production and removal) of the expressed proteins and organic metabolites, respectively, in a living system. The capacity to measure static levels of very large numbers of proteins and organic metabolites at one time, by use of mass spectrometric or 2-dimensional gel electrophoresis profiling techniques, has greatly advanced the field of Static Proteomics and Organeomics. Missing from all current proteomic and organeomic measurements is a key element, however: kinetics

or dynamic fluxes (i.e. rates of input and outflow of molecules, which brings in the dimension of time).

1) Differences from current mass spectrometric proteome profiling include:

- a) Current static mass spectrometric profiling techniques do not measure fluxes (kinetics or flow of molecules through pathways).
- b) The operational procedure of a prior step wherein stable isotope labels are administered *in vivo* or *ex vivo*, before collection of the biological sample, is not used or known in the field of profiling for proteomics and organeomics.
- c) The analytic procedure of monitoring particular mass isotopomers, measuring their quantitative abundances, and calculating individualized synthesis and turnover rates for each molecule based on its molecular formula, the stable isotope label added and mass isotopomer combinatorial calculations, has not been used in the field of proteomics and organeomics.
- d) By adding kinetics, the focus is changed fundamentally from concentrations of *individual molecules* to the control of *pathway fluxes* into and out of pools of molecules (i.e. to the true biochemical consequences of individual molecules on functional biochemical outputs).
- e) Kinetic measurements allow direct inference of regulatory steps controlling homeostasis of the proteome and organeome.

2) Fundamental advantages and/or surprising results that have emerged or may be expected:

- a) The translational (protein synthesis) program of a cell or organism can be immediately observed, without a lag phase for change in protein concentrations.
- b) The protein catabolic program of a cell or organism can be observed directly, which data is not otherwise available.
- c) The remarkable result has emerged that labeling of proteome has up to two orders of magnitude greater sensitivity than static measurements for detecting treatment effects (i.e. <200 proteins out of 20,000 show large changes in static

concentrations at steady state after even the most potent interventions, whereas up to 40-50% of proteins show large changes in synthetic or catabolic rates at steady state after potent interventions).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit and scope of the appended claims.

Applicants have not abandoned or dedicated to the public any unclaimed subject matter.

I claim:

1. A method of determining the molecular flux rates of a plurality of proteins in all or a portion of the proteome of a cell, tissue or organism, said method comprising:
 - a. administering one or more isotope-labeled protein precursors to said cell, tissue or organism for a period of time sufficient for one or more isotope labels to be incorporated into a plurality of proteins in the proteome or portion thereof of the cell, tissue or organism;
 - b. obtaining the proteome or portion thereof from the cell, tissue, or organism;
 - c. identifying a plurality of mass isotopomeric envelopes of ions representing individual proteins in the proteome or portion of the proteome by mass spectrometry;
 - d. quantifying relative and absolute mass isotopomer abundances of ions within the mass isotopomeric envelope corresponding to each identified protein by mass spectrometry; and
 - e. calculating the molecular flux rates of each identified protein to determine the molecular flux rates of said plurality of proteins.
2. The method of claim 1, wherein said administering step (a) is continuous.
3. The method of claim 1, wherein said administering step (a) comprises administering said one or more protein precursors at regular measured intervals.
4. The method of claim 1, wherein said one or more protein precursors are administered orally.
5. The method of claim 1, further comprising modifying said proteins prior to said measuring step.
6. The method of claim 5, wherein said modifying comprises biochemically degrading said proteins to peptides or individual amino acids.
7. The method of claim 6, wherein said modifying comprises chemically altering said proteins.

8. The method of claim 1, wherein said identifying step (c) further comprises identifying individual proteins in the proteome or portion of the proteome by chromatography.
9. The method of claim 1, wherein said plurality of proteins comprises the proteome of said cell, tissue, or organism.
10. The method of claim 1, further comprising the step of displaying the rates of synthesis and degradation of said plurality of proteins.
11. The method of claim 1, wherein said one or more protein precursors comprise an amino acid.
12. The method of claim 11, wherein the one or more protein precursors are selected from the group consisting of H_2O , CO_2 , NH_3 , and HCO_3 .
13. The method of claim 1, wherein said isotope label is selected from the group consisting of ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S and ^{34}S .
14. The method of claim 13, wherein said isotope is ^2H .
15. The method of claim 1, wherein said isotope-labeled protein precursor is selected from the group consisting of $^2\text{H}_2\text{O}$, H_2^{18}O , $^{13}\text{CO}_2$, $\text{C}^{18}\text{O}^{17}\text{O}$, H^{16}CO_3 , $^{15}\text{NH}_3$, ^2H -labeled amino acids, ^{13}C -labeled amino acids, ^{15}N -labeled amino acids, ^{18}O -labeled amino acids, ^{34}S -labeled amino acids, and ^{33}S -labeled amino acids.
16. The method of claim 15, wherein said isotope-labeled protein precursor is $^2\text{H}_2\text{O}$.
17. The method of claim 1, wherein the organism is a human.

18. The method of claim 1, comprising the additional step of discontinuing said administering step (a).
19. The method of claim 1, further comprising administering a diagnostic or therapeutic agent to said cell, tissue, or organism prior to said administering step (a).
20. A method of determining the effect of a diagnostic or therapeutic agent on a cell, tissue, or organism, comprising:
 - a. determining the molecular flux rates of a plurality of proteins in the cell, tissue, or organism according to the method of claim 1,
 - b. administering said agent; and
 - c. determining the molecular flux rates the plurality of proteins in the cell, tissue or organism according to the method of claim 1, to determine the effect of said diagnostic or therapeutic agent.
21. The method of claim 20, wherein said effect of a diagnostic or therapeutic agent on the molecular flux rates of the plurality of proteins in said cell, tissue or organism is used for the discovery, development or approval of a drug or other therapeutic agent.
22. A method of determining the effects of one or more genes on the molecular flux rates of a plurality of proteins in a cell, tissue, or organism, comprising:
 - a. determining the molecular flux rates a plurality of proteins in a first population of one or more cells, tissues, or organisms according to the method of claim 1, wherein said cells, tissues, or organisms of said first population comprise said one or more genes;
 - b. determining the molecular flux rates of the plurality of proteins in a second population of one or more cells, tissues, or organisms according to the method of claim 1, wherein said second population does not comprise said one or more genes;

- c. comparing the molecular flux rates in said first and second populations to determine the effect of one or more genes on the molecular flux rates of a plurality of proteins.
- 23. The method of claim 1, further comprising isolating a plurality of samples from said cell, tissue or organism.
- 24. A method of determining the molecular flux rates of a plurality of organic metabolites in all or a portion of the organeome of a cell, tissue or organism, said method comprising:
 - a. administering one or more isotope-labeled organic metabolites or organic metabolite precursors to said cell, tissue or organism for a period of time sufficient for one or more isotope labels from said one or more isotope-labeled organic metabolites to be incorporated into the a plurality of organic metabolites in the organeome or portion thereof of the cell, tissue or organism;
 - b. obtaining the organeome or portion thereof from the cell, tissue, or organism;
 - c. identifying a plurality of the mass isotopomeric envelopes of ions representing individual organic metabolites in the organeome or portion thereof by mass spectrometry;
 - d. quantifying relative and absolute mass isotopomer abundances of ions within the mass isotopomeric envelope corresponding to each organic metabolite identified in step (c) by mass spectrometry; and
 - e. calculating the rates of synthesis or removal of the identified organic metabolites to determine the rates of synthesis or removal of said plurality of organic metabolites.

25. The method of claim 24, wherein the one or more organic metabolites or organic metabolite precursors are selected from the group consisting of H_2O , CO_2 , NH_3 , HCO_3 , amino acids, monosaccharides, carbohydrates, lipids, fatty acids, nucleic acids, glycolytic intermediates, acetic acid, and tricarboxylic acid cycle intermediates.
26. The method of claim 24, wherein the isotope label is selected from the group consisting of ^2H , ^{13}C , ^{15}N , ^{18}O , ^{33}S or ^{34}S .
27. The method of claim 24, wherein said administering step (a) is continuous.
28. The method of claim 24, wherein said administering step (a) comprises administering said precursor at regular measured intervals.
29. The method of claim 24, wherein the one or more organic metabolites or organic metabolite precursors are administered orally.
30. The method of claim 24, comprising the additional step of discontinuing said administering step (a).
31. The method of claim 24, further comprising modifying said organic metabolites prior to said measuring step.
32. The method of claim 24, wherein said identifying step (c) further comprises identifying individual organic metabolites in the organeome or portion thereof by chromatography.
33. The method of claim 24, wherein said plurality of organic metabolites comprises the organeome of said cell, tissue, or organism.
34. The method of claim 24, further comprising the step of displaying the rates of synthesis or removal of said plurality of organic metabolites.
35. The method of claim 24, wherein the organic metabolite precursor is selected from the group consisting of H_2O , CO_2 , NH_3 , and HCO_3 .

36. The method of claim 26, wherein said isotope is ^2H .
37. The method of claim 35, wherein said organic metabolic precursor is H_2O .
38. The method of claim 24, wherein said isotope-labeled organic metabolite precursor is selected from the group consisting of $^2\text{H}_2\text{O}$, $^3\text{H}_2\text{O}$, and H_2^{18}O .
39. The method of claim 38, wherein said isotope-labeled organic metabolite precursor is $^2\text{H}_2\text{O}$.
40. The method of claim 24, wherein the organism is a human.
41. The method of claim 24, further comprising administering a diagnostic or therapeutic agent to said cell, tissue, or organism prior to said administering step (a).
42. A method of determining the effect of a diagnostic or therapeutic agent on a cell, tissue, or organism, comprising:
 - a. determining the rates of synthesis or removal of a plurality of organic metabolites in the cell, tissue, or organism according to the method of claim 24,
 - b. administering said agent; and
 - c. determining the rates of synthesis or removal of the plurality of organic metabolites in the cell, tissue or organism according to the method of claim 24, to determine the effect of said diagnostic or therapeutic agent.
43. The method of claim 42, wherein said effect of a diagnostic or therapeutic agent on the rates of synthesis or removal of the plurality of organic metabolites in said

cell, tissue or organism is used for the discovery, development or approval of a drug or other therapeutic agent.

44. A method of determining the effects of one or more genes on the molecular flux rates of a plurality of organic metabolites in a cell, tissue, or organism, comprising:
- a. determining the molecular flux rates of a plurality of organic metabolites in a first population of one or more cells, tissues, or organisms according to the method of claim 24, wherein said cells, tissues, or organisms in said first population comprise said one or more genes;
 - b. determining the rates of synthesis or removal of the plurality of organic metabolites in a second population of one or more cells, tissues, or organisms according to the method of claim 24, wherein said second population does not comprise said one or more genes;
 - c. comparing the rates of synthesis or removal in said first and second populations to determine the effect of one or more genes on the rate of synthesis or removal of a plurality of organic metabolites.
45. The method of claim 1, further comprising isolating a plurality of samples from said cell, tissue or organism.
46. The rights to drugs or other therapeutic agents so discovered by the method of claim 21.
47. The rights to drugs or other therapeutic agents so discovered by the method of claim 43.

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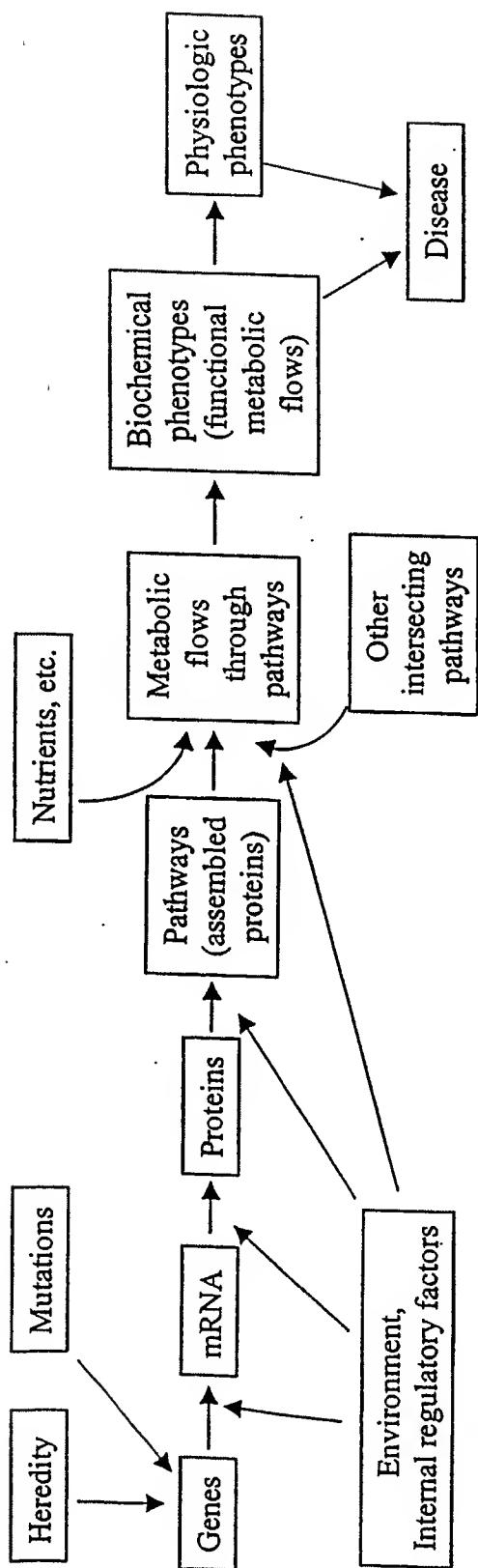


Fig. 1. From Gene to Protein to Phenotype and Disease: The Organization of Complex Biological Systems

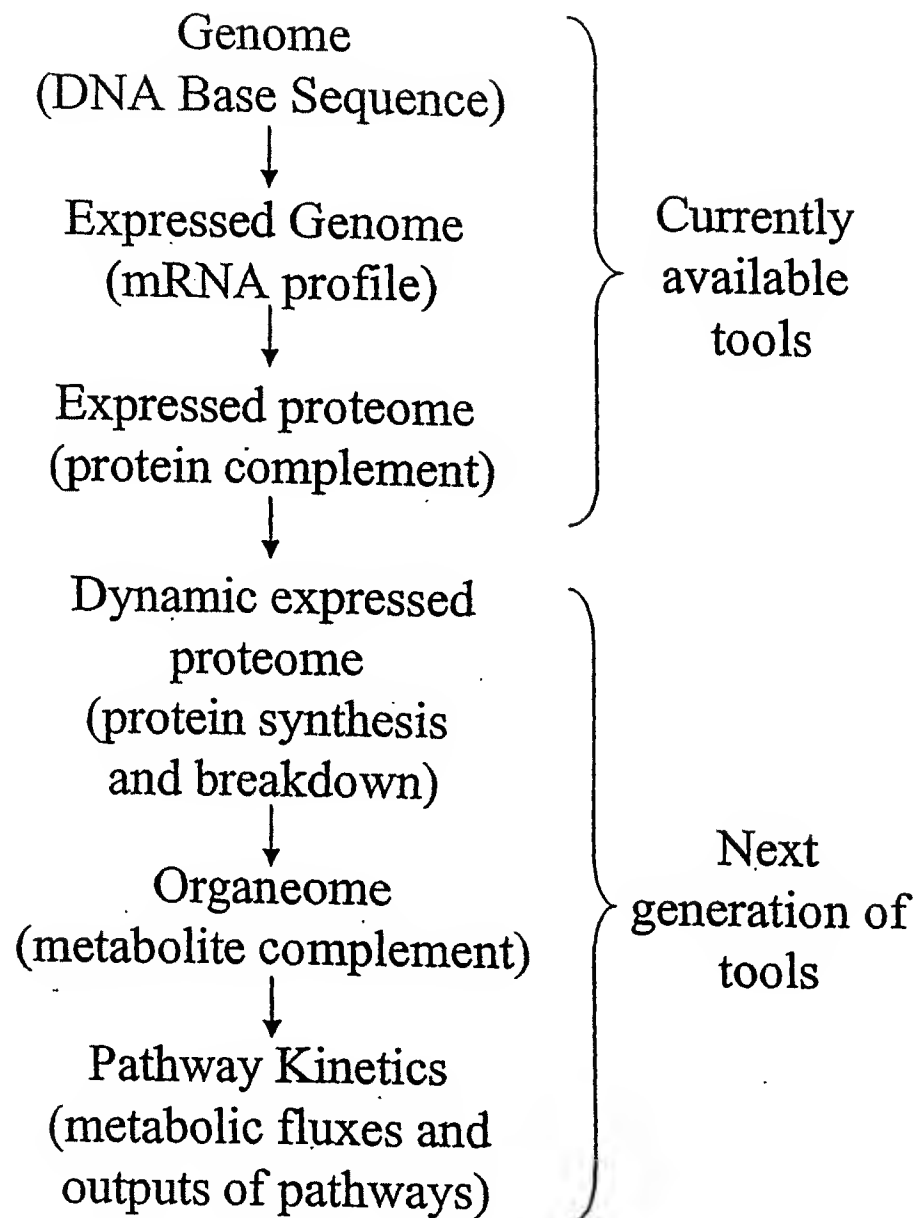


Fig. 2. Levels of “Functional Genomics”

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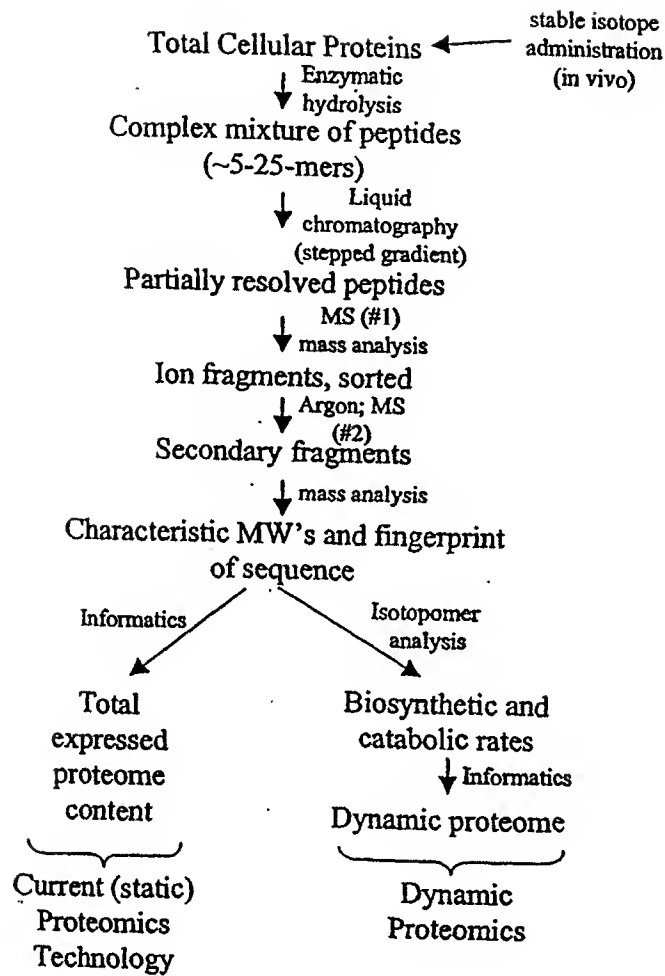


Fig. 3. Technique for Measuring Dynamic Proteomics

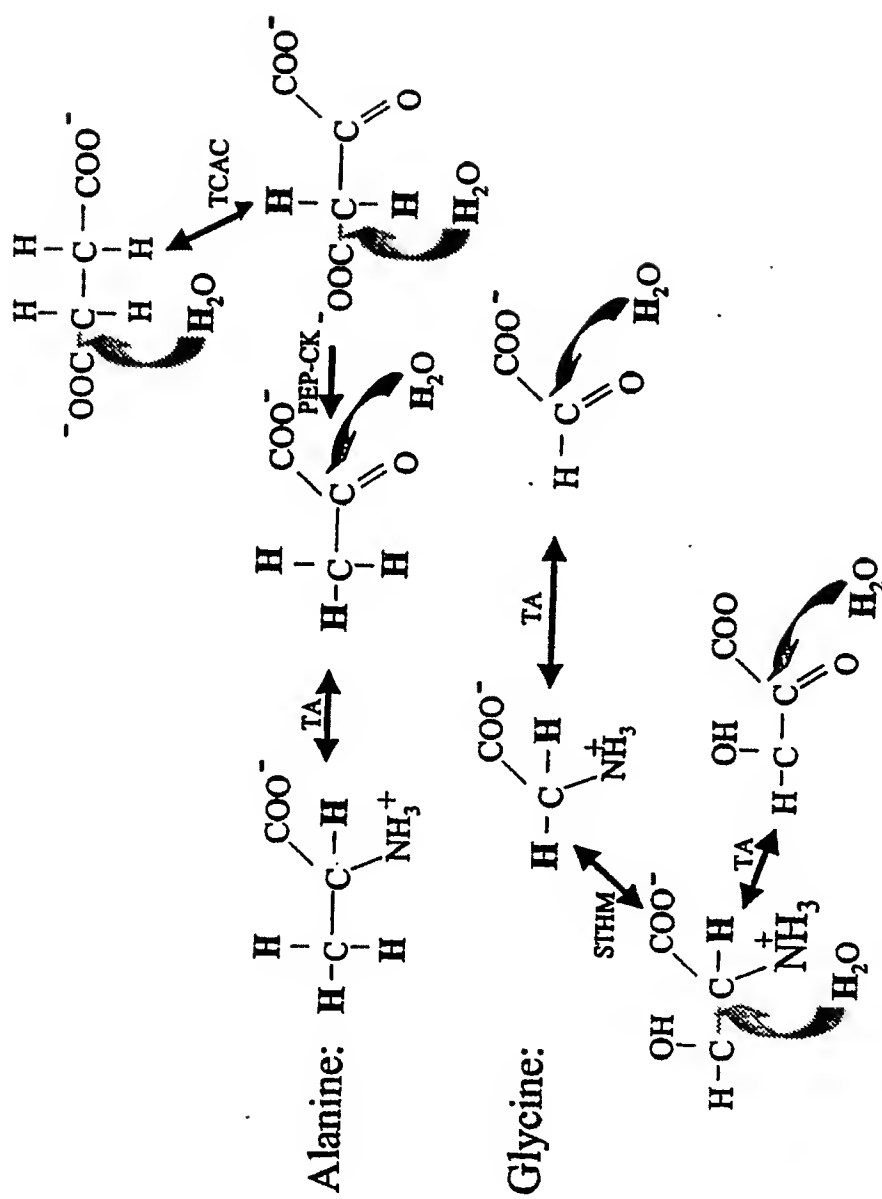


FIGURE 4A

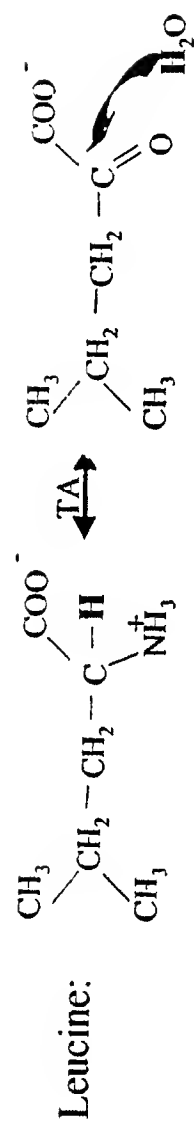


FIGURE 4B

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H_2^{18}O Labeling of Free Amino Acid for Protein Synthesis

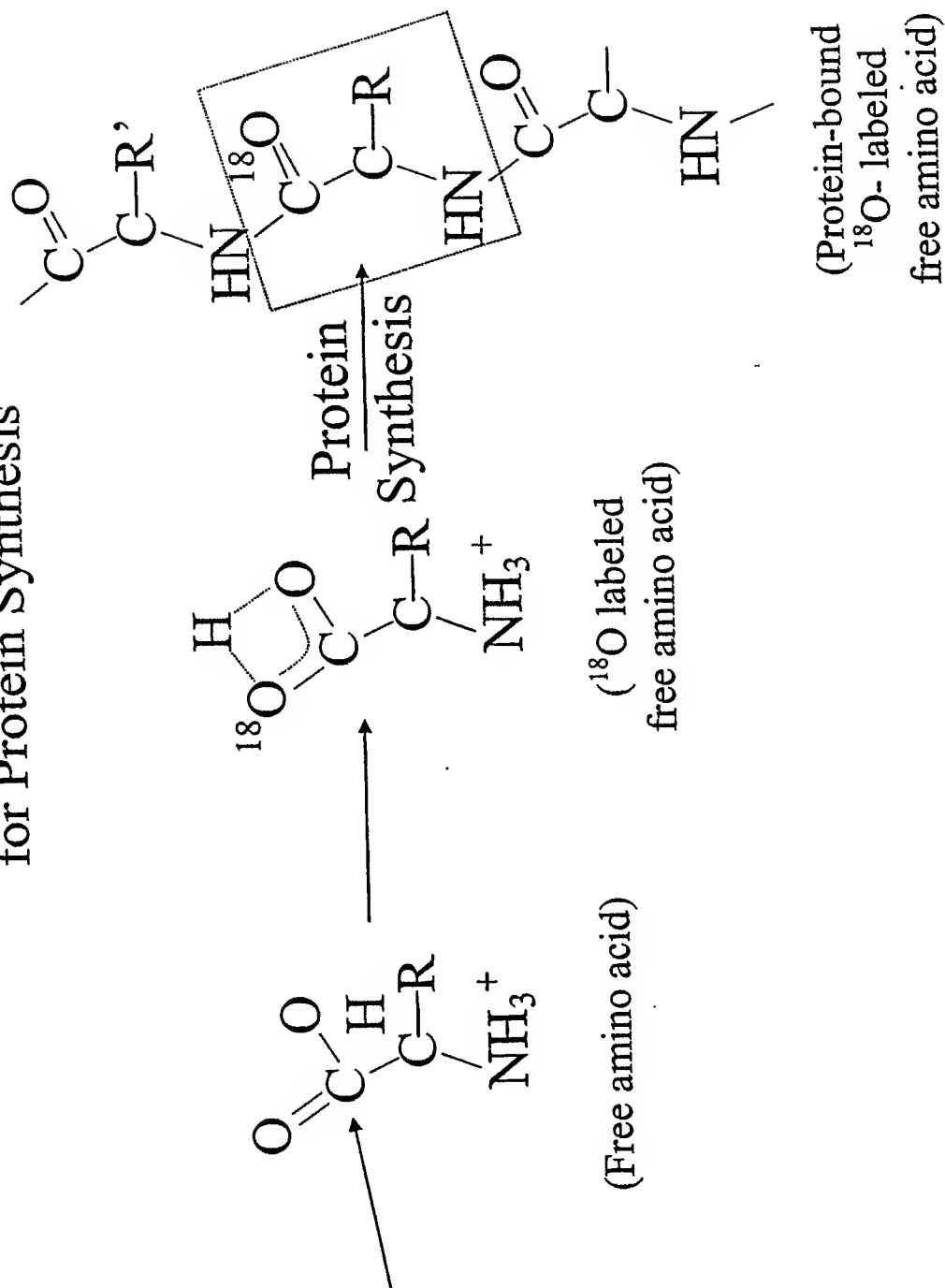


FIGURE 4C

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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR AUTOMATED, LARGE-SCALE MEASUREMENT OF THE MOLECULAR FLUX RATES OF THE
PROTEOME OR THE ORGANEOME USING MASS SPECTROMETRY

(57) Abstract: Disclosed here is a method for measuring the kinetics (i.e., the molecular flux rates - synthesis and breakdown or
removal rates) of a plurality of proteins or organic metabolites in living systems. The methods may be accomplished in a high-
throughput, large-scale automated manner, by using existing mass spectrometric profiling techniques and art well known in the fields
of static proteomics and static organomeomics, without the need for additional biochemical preparative steps or analytic/instrumental
devices.

WO 2004/011426 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/23340

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/02

US CL : 435/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, NPL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GYGI, S.P. et al. Using mass spectrometry for quantitative proteomics. Proteomics: A friends Guide. July 2000, pages 31-36, see entire document.	1-45
Y	VEENSTRA et al. Proteome Analysis Using Selective Incorporation of Isotopically Labeled Amino Acids. J. Am Soc Mass Spectrom. 2000, Vol. 11, pages 78-82, See entire document.	1-45
Y	ONG et al. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. Molecular and Cellular Proteomics. May 2002, Vol. 1, pages 376 - 386. See entire document.	1-45
Y	PAPAGEORGOPOULOS et al. Measuring Protein Synthesis by Mass Isotopomer Distribution Analysis (MIDA). Analytical Biochemistry. 1999, Vol. 267, pages 1-16, see entire document.	1-45
Y	PASA-TOLIC et al. High Throughput Proteome-Wide Precision Measurements of Protein Expression Using Mass Spectrometry. J. Am. Chem. Soc. 1999, Vol. 121, pages 7949-7950. See entire document.	1-45



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T"
"A" document defining the general state of the art which is not considered to be of particular relevance	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" documents referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

PCT/US03/23340

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHEVCHENKO et al. Rapid 'de Novo' Peptide Sequencing by a Combination of Nanoelectrospray, Isotopic Labeling and a Quadrupole/Time-of-Flight Mass Spectrometer. Rapid Comm. Mass Spectrom. 1997, Vol. 11, pages 1015-1024. See entire document.	1-45
Y	HANSEN et al. A Practical Method for Uniform Isotopic Labeling of Recombinant Proteins in Mammalian Cells. Biochemistry. 29 December 1992. Vol. 31, No. 51, pages 12713-12718, see entire document.	1-45
Y	BIER, D.M. Stable isotopes in biosciences, their measurement and models for amino acid metabolism. Eur J Pediatr. 1997. Vol 156 [Supp 1], pages S2-S8, see entire document.	1-45

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/23340

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claim Nos.: 46 and 47
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
because the claimed inventions are directed to non-statutory subject matter.
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.